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Long-term neuroprotective effect of erythropoietin on executive functions in very preterm children (EpoKids): protocol of a prospective follow-up study

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Abstract: **INTRODUCTION:** Premature infants are particularly vulnerable to brain injuries with associated cognitive and behavioural deficits. The worldwide first randomised interventional multicentre trial investigating the neuroprotective effects of erythropoietin (entitled 'Does erythropoietin improve outcome in very preterm infants?' (NCT00413946)) included 450 very preterm infants in Switzerland. MRI at term equivalent age showed less white matter (WM) injury in the erythropoietin group compared with the placebo group. Despite these promising imaging findings, neurodevelopmental outcome at 2 years showed no beneficial effect of early erythropoietin. One explanation could be that the assessment of more complex cognitive functions such as executive functions (EFs) is only possible at a later age. We hypothesise that due to improved WM development and fewer WM injuries, children born preterm treated with early erythropoietin will have better EF abilities at 7-12 years than those treated with placebo. **METHODS AND ANALYSIS:** 365 children who were included into the primary analysis of the original trial (NCT00413946) will be eligible in this prospective follow-up study at the age of 7-12 years. 185 children born at term will be control children. Primary outcome measures are EF abilities and processing speed, while secondary outcomes are academic performance, IQ, fine motor abilities and global brain connectivity. A comprehensive test battery will be applied to assess EFs. MRI will be performed to assess global brain connectivity. Cognitive scores and MRI measures will be compared between both groups using the Wilcoxon test. Propensity score matching will be used to balance gender, age, socioeconomic status and other potentially unbalanced variables between the children born preterm and the healthy control children. **ETHICS AND DISSEMINATION:** The cantonal ethical committee granted ethical approval for this study (KEK 2017-00521). Written consent will be obtained from the parents. Findings from this study will be disseminated via international and national conference presentations and publications in peer-reviewed journals.

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BMJ Open Long-term neuroprotective effect of erythropoietin on executive functions in very preterm children (EpoKids): protocol of a prospective follow-up study

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ABSTRACT

Introduction Premature infants are particularly vulnerable to brain injuries with associated cognitive and behavioural deficits. The worldwide first randomised interventional multicentre trial investigating the neuroprotective effects of erythropoietin (entitled ‘Does erythropoietin improve outcome in very preterm infants?’ (NCT00413946)) included 450 very preterm infants in Switzerland. MRI at term equivalent age showed less white matter (WM) injury in the erythropoietin group compared with the placebo group. Despite these promising imaging findings, neurodevelopmental outcome at 2 years showed no beneficial effect of early erythropoietin. One explanation could be that the assessment of more complex cognitive functions such as executive functions (EFs) is only possible at a later age. We hypothesise that due to improved WM development and fewer WM injuries, children born preterm treated with early erythropoietin will have better EF abilities at 7–12 years than those treated with placebo.

Methods and analysis 365 children who were included into the primary analysis of the original trial (NCT00413946) will be eligible in this prospective follow-up study at the age of 7–12 years. 185 children born at term will be control children. Primary outcome measures are EF abilities and processing speed, while secondary outcomes are academic performance, IQ, fine motor abilities and global brain connectivity. A comprehensive test battery will be applied to assess EFs. MRI will be performed to assess global brain connectivity. Cognitive scores and MRI measures will be compared between both groups using the Wilcoxon test. Propensity score matching will be used to balance gender, age, socioeconomic status and other potentially unbalanced variables between the children born preterm and the healthy control children.

Ethics and dissemination The cantonal ethical committee granted ethical approval for this study (KEK 2017-00521). Written consent will be obtained from the parents. Findings from this study will be disseminated via international and national conference presentations and publications in peer-reviewed journals.

Strengths and limitations of this study

- Long-term effects of early high-dose erythropoietin administration on executive function abilities and brain development will be investigated in children born very preterm.
- Long-term follow-up data on executive function abilities, processing speed and academic performance of a large cohort of very preterm children will be available through this study.
- Executive function abilities will be assessed comprehensively using standardised neuropsychological tests to measure core functions such as inhibition, cognitive flexibility, working memory and planning, and by investigating everyday life abilities as reported by the parents.
- Potential recruitment bias by the neurodevelopmental outcome of children born very preterm and by the socioeconomic status exists.
- Achieving the desired follow-up rate might prove challenging since the original randomised controlled trial was conducted between 2005 and 2012.

INTRODUCTION

Premature infants are particularly vulnerable to brain injury. As a consequence of perinatal brain injury, 5%–10% of survivors of premature birth develop cerebral palsy, and 40%–50% develop cognitive and behavioural deficits.^{1 2} A meta-analysis showed that children born very preterm manifest moderate to severe deficits in academic achievement, attention problems, internalising problems and poor executive functions.³ Executive functions refer to a family of top-down mental processes needed to attend to a task requiring concentration and attention.⁴ Three core executive functions have been described, namely, inhibition (the ability to suppress an automatic response), working memory (the

ability to manipulate information in short-term memory) and cognitive flexibility (the ability to switch between different mindsets, creatively thinking 'outside the box').⁵ Children born very preterm frequently perform worse in tests of inhibition, working memory and cognitive flexibility than age-matched term-born controls.^{3 6 7} Deficits across multiple executive function domains were also reported in adolescents^{8 9} and in adults born preterm.^{10–12} In addition to deficits in executive functions, processing speed has also been shown to be affected in adults born preterm.¹⁰ This is consistent with studies in children born preterm showing slower processing speed than term born children in infancy,^{13 14} toddlerhood¹⁵ and middle childhood.^{9 16 17} These impairments are likely to influence academic success. Indeed, it has been reported in school-aged children born very preterm that executive function deficits have a strong impact on learning skills⁹ and that processing speed and working memory abilities underlie academic attainment.^{18 19}

Associations between executive function deficits and cortical abnormalities have been reported in children and adults born very preterm.^{20–22} Structural alterations such as reduced grey and white matter volume accounted for 29% of the variance in executive functions in adolescents born very preterm at 12 years of age.²³ Also, delayed cortical maturational trajectories and significant associations between the change in cortical thickness and executive function scores were observed in adolescents born very preterm.²¹ Similarly, processing speed appears to be related to neonatal brain injury in very preterm children.²⁴

The high incidence of neurodevelopmental impairments including executive function deficits in very preterm infants is reflected in the ongoing search for neuroprotective interventions that can prevent brain injury or enhance repair of the immature brain, with the ultimate goal of improving long-term motor and cognitive outcome.²⁵ However, to date, no such postnatal neuroprotective intervention has been implemented into clinical management of very preterm infants. A neuroprotective intervention ideally addresses both the prevention of brain injury and subsequent failure of normal brain development.²⁶ Among several pharmacological candidates to prevent brain injury or to improve its development, erythropoietin has been shown to be one of the most promising neuroprotective agents.²⁷ Erythropoietin may exert its neuroprotective role by preventing acute injury via inhibition of glutamate release, modulation of intracellular calcium metabolism, induction of neuronal antiapoptotic factors, reduction of inflammation, decrease of nitric oxide-mediated injury and direct antioxidant effects.^{28 29} Furthermore, erythropoietin plays a role in developmental mechanisms, as it promotes proliferation and differentiation of preoligodendrocytes, and stimulates growth factors, such as vascular endothelial growth factor for increased angiogenesis, and brain-derived neurotrophic factor required for brain growth.²⁸

In Switzerland, the first interventional trial in very preterm infants using high-dose erythropoietin as a potential neuroprotective agent was initiated in 2005. It was a randomised, double-blind placebo-controlled, prospective multicentre trial entitled 'Does erythropoietin improve outcome in very preterm infants?' (NCT00413946) and has included 450 very preterm infants born below 32 weeks of gestation between 2005 and 2012.³⁰ Conventional MRI assessed in a subgroup of these infants showed that erythropoietin treatment was associated with a reduced risk of periventricular white matter injury, grey matter injury, white matter signal abnormalities and periventricular white matter volume loss, with a number needed to treat of seven preterm infants.³¹ Using tract-based spatial statistics of diffusion tensor imaging data, it was also shown that early high-dose erythropoietin improved white matter development at term equivalent age in these very preterm infants.³² Importantly, improvement of white matter development was seen globally and was consistent with findings from the conventional MRI analysis.³¹ At 2 years of age, neurodevelopmental outcome data were available for 365 children (81%). The mean mental and motor developmental indices as assessed by the Bayley Scales of Infant and Toddler Development-II were similar in both the erythropoietin and the placebo group.³⁰ In light of the positive effects of erythropoietin on brain development, the question arises why these effects did not translate into improved neurodevelopmental outcome at 2 years of age. Two possible explanations are that, first, the sample size might have been too small. This would be in line with the findings of a recent meta-analysis, comprising 1113 infants of four randomised controlled studies, showing that prophylactic erythropoietin improved the cognitive development at 18–24 months.³³ Second, merely developmental milestones can be measured at this young age while more complex cognitive functions such as executive functions are only developing,⁷ and hence the 2-year outcome might be used as a proof for safety of neuroprotective agents rather than their efficacy.³⁴ We therefore speculate that in our cohort of infants, early high-dose erythropoietin may produce benefits for cognitive development that become apparent only at a later age once higher-order cognitive functions develop and potential deficits may become evident. We hypothesise that the improved white matter development and the reduced rate of white matter injuries will be reflected in better executive functions and in improved processing speed at school age; hence, that early high-dose erythropoietin administration after birth will lead to improved executive functions and processing speed at school age in very preterm children.

HYPOTHESES AND AIMS OF THIS STUDY

The main aim of this project is to examine whether early administration of high-dose erythropoietin in very preterm infants has long-term beneficial effects on

executive functions, processing speed and global brain connectivity.

1. Very preterm children treated with early high-dose erythropoietin will have better executive function abilities and faster processing speed than those treated with placebo at the age of 7–12 years.
2. Global brain network connectivity is better in the children who were treated with early high-dose erythropoietin than in those treated with placebo.
3. There is a continuum in executive function and academic performance within term born children, children born very preterm treated with high-dose erythropoietin and children born very preterm allocated to placebo.
4. Improved longitudinal brain development between term equivalent age and 7–12 years ($n=165$) can be shown in those children who were treated with early high-dose erythropoietin compared with those treated with placebo.

METHODS AND ANALYSIS

Study participants

Very preterm children who participated in the trial entitled ‘Does erythropoietin improve outcome in very preterm infants?’ (NCT00413946) will be recruited at the age of 7–12 years. A total of 450 infants were enrolled and randomised (mean (range) gestational age, 29.0 (26.0–30.9) weeks; 264 (59%) female; mean (range) birth weight, 1210 (490–2290) g). Among them, 230 were allocated to the erythropoietin and 220 to the placebo group. Two infants in the erythropoietin group were excluded due to diagnosis of syndromes, which affect neurodevelopmental outcome ($n=448$). In the erythropoietin and placebo group, 57 infants received erythropoietin either in another dose as allocated or received supplemental erythropoietin and were therefore excluded. Further, 63 infants were excluded from the primary analysis (death or drop out), leaving 365 infants who were included into the primary analysis and therefore eligible for this study: 191 infants in the erythropoietin and 174 infants in the placebo group. A database of the erythropoietin trial including perinatal, socioeconomic and follow-up assessment data at 2 and 5 years of age has been established. Recruitment of the very preterm children will be done with an invitation letter sent by post and thereafter by telephone contact. In addition, 185 children born at term will be recruited as friends/siblings/peers of the preterm children or by advertisement through social media (www.epokids.ch) and at schools.

Primary and secondary outcome

The primary outcome measures of this study are executive functions and processing speed. The secondary outcome measures include academic performance, intelligence, fine motor abilities, brain development (global brain connectivity, network characteristics, brain volumes and biochemical profiles) assessed by MRI as well as

Table 1 Executive function abilities and processing speed

Assessed abilities	Applied assessment tools
Inhibition and interference	Stop-Signal Task (stop-signal reaction time) Color Word Interference Task (D-KEFS)
Working memory	Digit Span forward/backward (WISC-IV) Corsi Block Tapping Test TAP Working Memory (TAP 2.3)
Cognitive flexibility	Trail Making Task (D-KEFS) TAP Flexibility (TAP 2.3)
Planning	Tower Task (D-KEFS)
Fluency	Verbal Fluency (RWT) Design Fluency (D-KEFS)
Executive functions in everyday life	Parent rating (BRIEF questionnaire)
Processing speed	Coding, Symbol Search (both WISC-IV) Stop-Signal Task (mean reaction time of correct go trials)

Study-specific test battery to assess executive functions and processing speed.

BRIEF, Behaviour Rating Inventory of Executive Functions; D-KEFS, Delis-Kaplan Executive Function System; RWT, Regensburger Verbal Fluency Test (Regensburger Wortflüssigkeitstest); TAP, Test Battery for Attention Testing (Testbatterie zur Aufmerksamkeitsprüfung); WISC-IV, Wechsler Intelligence Scale for Children Fourth Edition.

behavioural problems, quality of life and variables of the family environment. The comparison of cognitive outcome measures and brain development between children born at term and children born very preterm is also a secondary outcome measure.

Primary outcome measures: executive functions and processing speed

Standardised neuropsychological tasks will be used to capture key aspects of executive functions, such as inhibition and interference, working memory, cognitive flexibility, planning, fluency and processing speed (table 1). Specifically, the stop-signal paradigm is a computerised tool for the investigation of response inhibition.³⁵ For this study, the task will be implemented in MATLAB (MathWorks) using the Psychtoolbox extensions.³⁶ All settings will be adopted from a previous study with very preterm children of a similar age.³⁷ In addition to the different measures of response inhibition, the mean reaction time of all correct go-trials will be used to assess processing speed. The ‘Test Battery for Attention Testing’ (Testbatterie zur Aufmerksamkeitsprüfung³⁸) consists of a set of computerised tests to selectively assess specific aspects of attentional processes. In this study, the flexibility and working memory subtests will be administered. The ‘Delis-Kaplan Executive Function System’³⁹ is a test battery designed exclusively for the assessment of executive functions, within both verbal and spatial modalities. For this

study, the Color-Word-Interference Task (inhibition), the Trail Making Task (cognitive flexibility), the Tower Task (visual-spatial planning) and the Design Fluency Test (fluency in generating visual patterns) will be used. The 'Regensburger Verbal Fluency Test' (Regensburger Wortflüssigkeitstest⁴⁰) is a German language verbal fluency test, and phonetic and semantic subtests will be used for this study. The 'Corsi Block Tapping-Test'⁴¹ is a well-validated tool often applied in clinical practice and experimental research. The forward and backward conditions will be used to assess visual-spatial short-term and working memory, respectively. The following subtests of the 'Wechsler Intelligence Scale for Children, Fourth Edition' (WISC-IV, German version⁴²) will be used: the Digit Span subtest will be used to assess verbal short-term, and working memory and the Symbol Search and Coding subtests will be used to assess processing speed. The 'Behaviour Rating Inventory of Executive Functions' (BRIEF⁴³) is a questionnaire designed to gather information about an individual's use of executive function skills in daily life (at home and in school). Parents will complete this questionnaire.

Secondary outcome measures

Intelligence, fine motor abilities and academic performance

Intelligence will be estimated with four subtests of the WISC-IV⁴²: Block Design, Similarities, Digit Span and Coding. This subtest combination has been shown to correlate highly with the full version ($r > 0.90$).⁴⁴ To assess fine motor abilities, the Pegboard and Sequential Finger Movements subtests of the Zurich Neuromotor Assessment⁴⁵ will be used. Due to the lack of a comprehensive test battery to assess academic abilities appropriate for the Swiss school system (such as for example the Wechsler Individual Achievement Test-II⁴⁶), in this study, subtests of different validated instruments will be applied to assess math, reading and spelling skills: mathematical abilities will be tested with the Subtraction and Completion subtests of the 'Heidelberger Computing Test' (Heidelberger Rechentest)⁴⁷ and the Applied Math subtest of the 'Adaptive Intelligence Diagnostic Tool', Third Edition (Adaptives Intelligenzdiagnostikum (AID-3)).⁴⁸ Reading skills will be tested with the 1-min Reading Fluency subtest of the 'Salzburger Reading and Writing Test-II' (Salzburger Lese-/Rechtschreibtest-II)⁴⁹ and the Text Comprehension subtest of the 'Salzburger Reading Screening' (Salzburger Lesescreening⁵⁰). Spelling skills will be tested with the Spelling subtest of the 'Hamburger Writing Test' (Hamburger Schreibprobe⁵¹).

This test battery will be divided into two sessions: the first testing session will be done in the morning (approximately 2 hours) and the second half (approximately 1.5–2 hours) after a lunch break, followed by the MRI at the end of the day. The executive function tests and the tests to assess school skills will be undertaken in a randomised order to avoid fatigue effects.

Questionnaires

In addition to collecting cognitive and neuroimaging data from the participants, they will also complete two questionnaires to report on their quality of life and their relationship with their parents. The parents will be asked to complete a set of questionnaires to provide detailed information on aspects of their children's behaviour and quality of life and on their own health and well-being, their relationship with their child and various aspects of the family situation. Table 2 provides an overview of the applied questionnaires.

Magnetic resonance imaging

Cerebral MRI will be performed on a 3T GE MR750 scanner. Hearing protection will be provided with earplugs and headsets. The heart and respiratory rate will be monitored continuously. Image analysis will focus on group differences in the global network connectivity, topology and brain development. For this purpose, diffusion tensor imaging will be performed (TE/TR=77/6500 ms, 35 gradient sampling directions, 4 B0 images, field of view (FOV): 27.8 cm, acquisition matrix: 96×96). Furthermore, for volumetric analysis 3D T1 SPGR (TE/TR=5/11 ms, inversion time (TI)=600 ms, flip angle: 8°, FOV=25.6 cm, reconstruction matrix: 256×256, 158 slices/slab) and 3D T2 CUBE (TE/TR=98/2800 ms, FOV=256 cm, matrix: 256×256) sequences will be acquired. Resting-state functional MRI (TE/TR=32/1925 ms, flip angle=74°, FOV=27.8 cm, matrix=64×64) will be acquired for analysing the functional connectivity between the groups. Magnetic resonance spectroscopy (TE/TR 35/3000 ms) will be acquired with voxels placed in basal ganglia/thalami and frontal white matter, respectively, to look at group differences in the biochemical profile. Furthermore, T2 relaxometry will be performed using a dual echo PD/T2 FSE sequence (TE₁/TE₂/TR=30/118/7000 ms, echo train length=16, FOV=27.8, matrix=412×224 (interpolated to 512×512)) in order to look at group differences in T2 times in the grey and white matter. Longitudinal brain growth can be analysed in the subgroup of infants in which imaging was acquired at term equivalent age (n=165).³¹

Comparison of cognitive measures and global brain connectivity between children born at term and children born very preterm

Cognitive scores and measures of global brain network connectivity will be compared between the children born very preterm and those born at term to test whether there is a continuum in executive function performance and connectivity within term born children, children born very preterm treated with early high-dose erythropoietin and children born very preterm treated with placebo (see the Statistics section).

Study design and power calculation

Study design

This is a prospective follow-up project of the randomised erythropoietin trial NCT00413946. Recruitment process: 450 very preterm infants were randomised into the erythropoietin trial between 2005 and 2012. Of these, the 365 children

Table 2 List of applied questionnaires

Assessed domains and subdomains	Questionnaire	Completed by*
Information on child		
Information on health and academic outcome	Detailed information on schooling, health concerns and leisure activities	Parents
Behavioural screening: emotional symptoms, conduct problems, hyperactivity/inattention, peer relationship problems, prosocial behaviour	Strength and Difficulty Questionnaire ⁵⁵	Parents
Autistic traits screening	Social and Communication Disorder Checklist ⁵⁶	Parents
Health-related quality of life: physical, psychological well-being, autonomy and parent relation, peers and social support, school environment	Kidscreen-27 ⁵⁷	Parents and children
Information on parents		
Executive function abilities relevant for everyday life: inhibit, shift, emotional control, self-monitor, initiate, working memory, plan/organise, task monitor and organisation of materials	Behaviour Rating Inventory of Executive Functions-Adult version ⁵⁸	Parents
Physical and mental health: vitality, physical functioning, bodily pain, general health perceptions, physical role functioning, emotional role functioning, social role functioning, mental health	Short Form Health Survey ⁵⁹	Parents
Information on family		
Family socioeconomic status	Detailed information on for example, parental age, nationality, marital status, first language, maternal and paternal education and occupation, current living arrangements and financial situation	Parents
Quality of family environment: family cohesion, expressiveness, conflict	Family Relationship Index ⁶⁰	Parents
Perceived social support for the family	Social Support Questionnaire (F-SozU-14) ⁶¹	Parents
Parenting: positive parenting behaviour, involvement, (poor) monitoring, inconsistent discipline, authoritarian parenting, responsible parenting, corporal punishment	German version of the Alabama parenting Questionnaire for elementary school children (DEAPQ-EL-GS) ⁶²	Parents
Quality of parent-child interaction: cohesion, identification, autonomy, conflicts, rejection/neglect, punishment, emotional burden, fears/overprotection, aid	Parental-Representation-Screening-Questionnaire (Elternbild-Fragebogen, EBF-KJ) ⁶³	Children
Critical life events	12-item list of critical life events	Parents

*Questionnaires are completed by at least one parent.

who were included into the primary analysis are eligible and will be contacted for this follow-up study. These children will be between 7 and 12 years old during the project time and are, hence, eligible for this study.

Power calculation: The primary outcome of this study will be assessed by the executive function tasks described above. Based on the literature, we expect a minimal effect size of 0.36 (since effect sizes for executive outcome measures range from 0.36 for working memory, 0.49 for cognitive flexibility and 0.57 for verbal fluency³ when comparing very preterm to term-born children). Considering the smallest effect size of 0.36, a minimal sample size of 123 children/participants per group is required, assuming a two-sided alpha error of 0.05 and a power (1-beta) of 0.8. To compensate for drop-outs and considering that the effect size between preterm

groups (erythropoietin vs placebo) might be smaller than between preterm participants and their term-born peers, we will add 20% to each group, targeting at least 148 children/participants per group, in total 296 children. There might be some imbalance in the distribution of gestational age, socioeconomic status or gender in the children willing to participate in this follow-up study between treatment groups. These imbalances will be taken into account in the analysis.

Statistics

Descriptive statistics include mean and SD or median and IQR for the continuous or ordinally scaled variables. Numbers and percentages of total will be reported for the categorical variables. Unpaired Wilcoxon tests or

t-tests for ordinally scaled or continuous variables and χ^2 or Fisher's exact test for dichotomous outcomes will be used. Linear regression models will be used to examine differences between the groups in executive function abilities. The independent variable in all regression models will be group status (erythropoietin vs placebo) and potentially other variables such as gender, gestational age and socioeconomic status. P values <0.05 will be considered statistically significant. Brain network features will be analysed using graph theory measures, characterising network structure and function.^{52–53} Network description will include measures of topological organisation (modularity, small-world and rich-club indices), integration and segregation. Cognitive scores and measures of global brain network connectivity and topography will be compared between the erythropoietin and placebo group using the Wilcoxon test. The statistical programming language R⁵⁴ for Windows will be used for all analyses.

In a secondary analysis, the 185 term-born control children, primarily recruited for the evaluation in this study, will be compared with the children born very preterm included in the erythropoietin trial. Propensity score matching will be used to balance gender, age, socioeconomic status and other potentially unbalanced variables between the children born very preterm and the term-born control children. The aim of this approach is to balance other factors, enabling the quantification of the effects of being born very preterm, and within subgroups of children with or without erythropoietin.

ETHICS AND DISSEMINATION

Written informed consent will be obtained from the parents. Data handling, record keeping and archiving will be done according to the guidelines given by the ethical committee. The results of this study will be published in peer-reviewed journals, and the findings will be presented at national and international conferences for widespread dissemination of the results.

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Contributors CFH, FW, RTOG, UH and BL were involved in the study design. FW, BS, VD and CFH were responsible for conceiving ethical approval. CFH, FW, RTOG and UH wrote the first draft of the manuscript. CFH, FW, RTOG, UH, BL, PH, J-CF, VD and BS commented and critically reviewed drafts of the paper.

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Competing interests None declared.

Patient consent Parental/guardian consent obtained.

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